[25] Gene Transfer to the Brain Using Feline Immunodeficiency Virus-Based Lentivirus Vectors

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Introduction

Neurodegenerative diseases of the central nervous system (CNS) can display restricted or widespread CNS pathology. Examples of disorders demonstrating extensive CNS involvement include Alzheimer's disease,[1] the neuronal ceroid lipofuscinoses (CLN or NCL),[2,3] and the mucopolysaccharidoses (MPS types I to VII).[4] Disorders with relatively restricted neuronal cell loss include Parkinson's disease[5] and motor neuron diseases such as amyotrophic lateral sclerosis (ALS)[6] and the spinocerebellar ataxias (SCA).[7] For many neurodegenerative diseases, gene therapy approaches are being investigated, and may prove viable treatment options. Studies in our own laboratory include investigation of CNS gene transfer strategies for treatment of CLN, MPS, ALS, and the SCA. The CLN and MPS are inherited lysosomal storage diseases characterized by abnormal lysosomal storage deposits. CNS manifestations of these diseases include advancing neuronal dysfunction resulting in progressive cognitive and visual deterioration. ALS is characterized by degeneration of motor neurons in the spinal cord and brain stem, followed by further degeneration of corticospinal neurons in the cerebral cortex. ALS patients manifest increasingly severe muscular weakness and eventual death due to neuromuscular respiratory failure. Approximately 5% of ALS is caused by mutations in superoxide dismutase (SOD1) while 95% are of unknown origin. The SCA are inherited disorders characterized by selective loss of cerebellar neurons leading to motor dysfunction and ataxia.

Gene transfer strategies are often based on delivery of a correct cDNA copy of the affected gene to the relevant cells *in vivo*. For many of the lysosomal storage diseases, the affected gene encodes a soluble lysosomal protein that when overexpressed can be secreted and taken up by neighboring cells via mannose or

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mannose 6-phospate receptors. As a result, transduction of a small proportion of CNS cells can potentially mediate widespread correction. The task is more difficult with a membrane-integral lysosomal protein, where prevention and/or reversal of neurodegeneration may necessitate delivery of the gene to most affected CNS cells.

In instances where the defective protein performs a dominant negative function, or deleterious function, provision of a normal version of the gene may be without beneficial effect. Several of the SCA fall into this category; expanded polyglutamine tracts create a toxic gain of function in the encoded protein.[7] Similarly the SOD1 mutation in ALS is a gain of function mutation leading to accumulation of toxic reactive metabolites.[6] Gene transfer approaches for such disorders are aimed at either down-regulating expression of the mutated gene or countering its toxic effects. Neuroprotective strategies such as transfer of genes expressing neurotrophic factors or anti-apoptotic molecules could be undertaken to prevent or delay neuronal cell death, and may find general application to neurodegenerative diseases.[8-13]

Targeting of neurons requires use of a vector that can transduce fully differentiated nondividing cells. *In vivo* rodent studies have found that although recombinant adenoviruses based on serotype 5 can infect neurons *in vivo,* their preference is for astrocytes,[14-16] and the vector-associated inflammatory response often curtails transgene expression.[15,17] Commonly used retroviral vectors based on oncoretroviruses show strict requirements for cell division,[18] and thus are not be useful for targeting neurons. Retroviral vectors based on lentiviruses, such as human immunodeficiency virus (HIV) or feline immunodeficiency virus (FIV), possess nuclear import mechanisms and demonstrate ability to transduce nondividing, fully

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differentiated cell types including postmitotic neurons, *in vitro* and *in vivo*.[15,19–21] Lentiviral vector gene transfer to the brain is accompanied by minimal if any vector-associated inflammatory response, and transgene expression is stable.[15] Thus lentivirus-based vectors show promise for neuronal gene transfer. Our recent studies have investigated the use of recombinant FIV-based vectors for direct gene transfer to the MPS VII mouse brain cerebrum[22] as a model for gene therapy application for widespread neurodegenerative diseases. We have also investigated the feasibility of recombinant FIV as a vector for gene transfer to cerebellar neurons,[14] for potential therapeutic use in degenerative diseases of the cerebellum.

Wild-type FIV causes an immunodeficiency disease in cats and, despite prevalent exposure, is not known to cause infection or disease in humans.[23] Like other retroviruses, FIV is an enveloped virus, with a single stranded RNA genome. Cell entry of enveloped viruses is a fusogenic process directed by the virus envelope protein, resulting in delivery of nucleocapsids either directly into the cytoplasm or into intracellular vesicular compartments. Reverse transcription, mediated by the retroviral enzyme reverse transcriptase (RT), then proceeds to generate double-stranded DNA. In oncoretroviruses, such as the Moloney murine leukemia virus (MLV, used commonly in recombinant form as a gene transfer vector), access of the double-stranded DNA preintegration complex to the host cell genome requires dissolution of the nuclear membrane, as occurs during cell mitosis. In contrast, lentiviral preintegration complexes are equipped to mediate nuclear import, thus enabling integration of genetic material into the genomes of nondividing as well as dividing cells.

The native FIV genome contains the basic retroviral *gag, pol,* and *env* open reading frames (Fig. 1A) for production of matrix and nucleocapsid proteins, RT, polymerase and integrase proteins, and envelope proteins, respectively.[24] The FIV genome is simpler than the HIV genome, and encodes only three accessory proteins: vif, orf2, and rev.[25] Reports by Poeschla *et al.*,[20] Johnston *et al.*,[19] and Curran *et al.*[26] describe construction of recombinant FIV gene transfer vectors that can be produced at high particle titer and are able to efficiently transduce dividing and nondividing cells. These protocols utilize a triple plasmid transfection system. The packaging plasmid provides all the necessary viral proteins in

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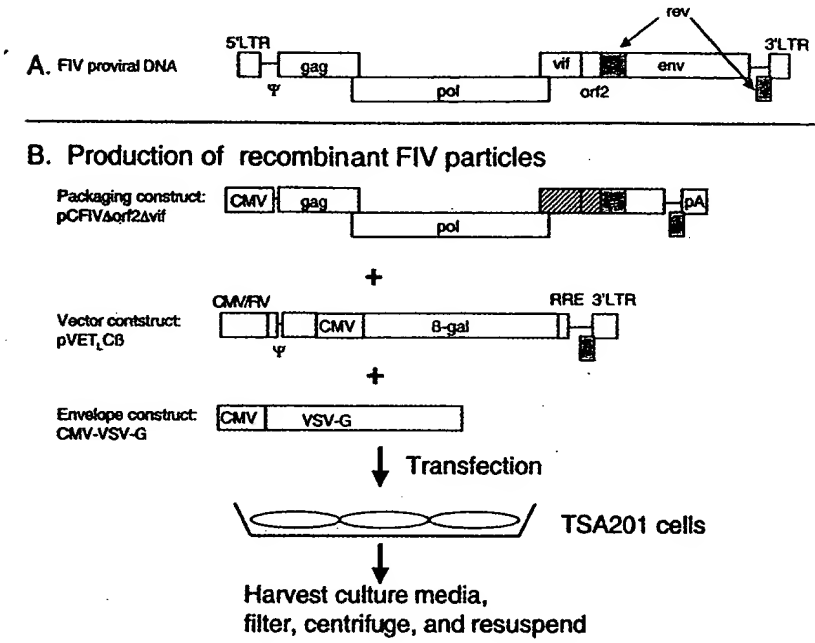

FIG. 1. Recombinant FIV particle production. (A) FIV proviral DNA contains three large open reading frames (*gag, pol,* and *env*), and three small regions encoding accessory proteins (*vif, orf2,* and *rev*). The packaging signal (Ψ) extents from noncoding sequences just downstream of the 5′ LTR to coding sequences within the 5′ portion of *gag*. (B) For particle production, TSA201 cells are transfected with packaging, vector, and envelope plasmids. Full-length transcripts from the vector plasmid contain an intact packaging signal and are packaged into enveloped particles. At 24, 36, and 72 h posttransfection the culture media (containing particles) is harvested and filtered, and the particles are concentrated by centrifugation.

trans (except for the env protein), but it is not packaged into particles. The vector plasmid contains the transgene expression cassette and retains the minimal *cis*-acting viral sequences to allow efficient genome packaging, reverse transcription, and integration. The third plasmid encodes a chosen envelope protein in *trans*. Most lentiviral vector studies to date have used recombinant vectors pseudotyped with the envelope glycoprotein from vescicular stomatitis virus (VSV-G), because it imparts particle stability and mediates widespread cellular tropism.[27] This chapter outlines techniques for (1) production of VSV-G-pseudotyped recombinant FIV particles encoding the reporter protein bacterial β-galactosidase (FIVβgal), (2) vector injection in mouse brain striatum and cerebellum, and (3) immunofluorescent staining for determination of cell types transduced.

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Materials

Recombinant FIV Particle Production

Plasmids: packaging construct, vector construct, and envelope construct (Fig. 1B): The FIV packaging construct (pCFIVΔorf2Δvif)[19] was derived from the FIV molecular clone p34TF10. The packaging construct retains full-length *gag* and *pol*, and *rev*, but contains a deletion in the *env* gene, and mutations in *vif* and *orf2* genes. The vif and orf2 accessory proteins have been determined to be dispensable both for recombinant particle production and for transduction of brain *in vivo*. The third accessory gene, *rev*, is retained since rev is necessary for efficient nuclear export of long and full-length transcripts. The native 5′ LTR has been replaced by the human cytomegalovirus (CMV) immediate early promoter/enhancer, and the 3′ LTR has been replaced with the simian virus 40 polyadenylation signal. The vector construct ($pVET_LC\beta$)[19] carries the transgene of interest driven by an internal promoter. Here the *lacz* gene, encoding the reporter protein bacterial β-galactosidase, is driven by the CMV promoter. The 5′ U3 of FIV has been replaced with the CMV promoter. $pVET_LC\beta$ has been deleted of all viral coding regions with the exception of a 5′ portion of *gag* (which contains part of the packaging signal). Last a third plasmid, the envelope construct, provides the envelope protein in trans. Here we use a plasmid encoding the VSV-G directed by the CMV promoter.[28]

TSA201 cells[29] maintained in exponential growth in DMEM-10. TSA201 cells were derived from 293 human embryonic kidney epithelial cells (ATCC CRL-1573) and grow as monolayers. They are easily lifted with trypsin for passage.

Incubator at 37° with 5% CO_2

HEPES buffered saline (HBS):

HEPES	5.0 g
NaCl	8.0 g
KCl	0.37 g
$Na_2HPO_4 \cdot 7H_2O$	0.188 g
Glucose	1.0 g

Bring to 1 liter in ddH_2O, pH to 7.1 with concentrated NaOH, filter sterilize and store at 4°

2.5 *M* $CaCl_2$

DMEM: Dulbecco's modified Eagle's medium

DMEM-10: DMEM with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin

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DMEM-2: DMEM with 2% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin
Bottle-top filters (500 ml capacity, Nalgene PES low protein binding)
Lactose buffer: phosphate-buffered saline (PBS), pH 7.4 (Sigma P-3813) with 40 mg/ml lactose, filter-sterilized
Sorvall centrifuge RC 26 Plus, with SLA 1500 rotor
Sorvall centrifugation bottles (250 ml capacity)

Determination of Transduction Titer by X-Gal Staining

HT-1080 cells (ATCC CRL-121) maintained in exponential growth in DMEM-10. These cells are derived from human fibrosarcoma and grow as monolayers. They are easily lifted with trypsin for passage.

Incubator at 37° with 5% CO_2
Six-well tissue culture dishes
DMEM-2 and DMEM-10 (see above)
Polybrene stock: 8 mg/ml in ddH_2O, filter sterilized
DMEM-2/Polybrene: On the day of use, dilute the Polybrene stock 1/2000 in DMEM-2, for a final Polybrene concentration of 4 μg/ml
Dilution tubes (3.5 ml polystyrene sterile tubes)
1% glutaraldehyde in PBS
KC Mixer:

35 m*M* K_3 $Fe(CN)_6$	5.74 g
35 m*M* K_4 $Fe(CN)_6 \cdot 3HO$	7.35 g
2 m*M* $MgCl_2$	1 ml of 1 *M* stock
0.01% sodium desoxycholate	0.5 ml of 10% stock
0.02% NP40	1.0 ml of 10% stock

Add to PBS for a final volume of 500 ml. Do not add the $MgCl_2$ until the previous ingredients have dissolved. Filter through a 0.45 μm bottle top filter and store in the dark at 4°.
X-Gal (5′-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) stock: Dissolve at 40 mg/ml in *N*′,*N*-dimethyl formamide, and store at −20° (does not freeze).
X-Gal solution (staining solution): Dilute X-Gal stock to 1 mg/ml in the KC mixer just prior to use. Prewarming separately the KC mixer and the X-Gal stock at 37° before mixing can help to avoid precipitation that can occur with longer incubation times.

Vector Injection into Adult Mouse Striatum or Cerebellum

Ketamine/xylazine mix: combine 8.9 ml of sterile PBS with 1 ml of 100 mg/ml ketamine and 0.1 ml of 100 mg/ml xylazine for final concentrations of 10 mg/ml ketamine and 1 mg/ml xylazine.

Insulin syringe with attached 28-gauge needle
Underpads (blue chucks)
Eye ointment (bacitracin zinc and polymyxin B sulfate ophthalmic ointment)
Razor to shave mouse head
Iodine tincture
Surgical instruments (autoclaved): scalpel and blades, curette, forceps, scissors, hemostat
Dry sterilizer (Germinator 500, Cellpoint Scientific, Inc.)
Small sterile beaker with 70% ethanol
Surgical suture (4.0 silk) with attached needle (1/2 × 17 mm)
Small animal stereotaxic frame with mouse adaptor (KOPF Instruments)
Microprocessor-controlled pump (World Precision Instruments UltraMicroPump) mounted onto the stereotaxic frame
Microprocessor-based controller (World Precision Instruments Micro 1 model)
Small box (we use tip box) taped to the base of the stereotaxic frame, to lay the mouse on
10 μl glass Hamilton syringe with a removable stainless steel blunt-ended 33-gauge needle (Hamilton) (for striatal injection)
5 μl glass Hamilton syringe with a pulled glass microcapillary tube attached (for cerebellar injection): the microcapillary tubes are pulled out on microcapillary needle puller, and cemented to the syringe with Superglue
Drill (Dremel Moto-Tool Model 395 Type 5, or equivalent) with 003 bit
Dissecting microscope (optional)
Lactated Ringer's
3 ml syringe with 25-gauge needle
Recovery mouse cage with clean towel bedding
Lamp with 75 watt bulb, suspended 2.5 feet above the recovery cage
Mouse brain atlas (*The Mouse Brain in Stereotaxic Coordinates,* K. B. I. Franklin and G. Paxinos, Academic Press, 1997)
FIVβgal: concentrated recombinant FIV particles encoding the β-galactosidase transgene, kept on ice

Animal Perfusion/Fixation

Ketamine/xylazine mix (see recipe above)
PBS
2% paraformaldehyde in PBS: in a fume hood, add 2 g paraformaldehyde (powder grade) to near 100 ml of PBS. Cover and heat to 50–60° with stirring. Do not boil. Add a few drops of concentrated NaOH to help dissolution, and bring pH back to 7.4 with concentrated HCl. Bring final volume to 100 ml with PBS. Filter through a 0.45 μm bottle top filter and cool on ice. Store at 4°. Use within 1 week of preparation.

Peristaltic perfusion pump
Flexible rubber hosing 2–3 mm in diameter
Butterfly IV catheter, 23 or 25 gauge
Instruments: scissors, forceps
Styrofoam board
Spray bottle with 70% ethanol

Dissecting Out the Brain

Razor blade or scalpel
Ronguers (Roboz Surgical Instruments)
Curette (spoonlike instrument)
30% Sucrose in PBS
Plastic molds (Peel-a-ways from VWR Scientific Products)
OCT (Tissue-Tek)
Dry ice/95% ethanol bath

Tissue Sectioning

Cryostat
Glass slides (Fisher Superfrost glass plus microscope slides)
Slide boxes
For thick, free-floating sections:
  Forceps
  24-well tissue culture dishes
  PBS
  PBS/azide: PBS with 0.02% sodium azide

Dual Immunofluorescent Staining

Staining of Cryosections on Glass Slides
Humidity chamber (wet paper towels in closed shallow container)
PAP pen (Electron Microscopy Sciences)
PBS
BSA: bovine serum albumin (Sigma, ELISA grade)
Block: PBS with 10% normal (goat) serum (from same species as the secondary antibody), 0.1% Triton X-100, and 0.02% sodium azide (can use 3% BSA in place of or in addition to the serum)
Primary antibody diluent: 3% BSA in PBS with 0.1% Triton X-100 and 0.02% azide
Wash buffer: PBS containing 1% normal (goat) serum, 0.1% Triton X-100, and 0.02% azide
Secondary antibody diluent: PBS containing 1% normal (goat) serum, 0.1% Triton X-100, and 0.02% azide

Primary antibodies:
Polyclonal rabbit anti-β-galactosidase (BioDesign B59136R, 10 mg/ml). Dilute 1/1500.
Mouse mAb to NeuN (Chemicon MAB377, 1 mg/ml). Dilute 1/200.
Mouse mAb to GFAP (Sigma C9205, 1 mg/ml, Cy3 conjugate). Dilute 1/2000.
We purchase this antibody directly conjugated to Cy-3; it works well and eliminates the need for a secondary antibody.
Mouse monoclonal antibody to calbinbin-D-28K (Sigma, ascites). Dilute 1/3000.
Secondary antibodies:
Goat anti-rabbit Alexa 488 (Molecular Probes). Dilute 1/200.
Goat anti-mouse IgG lissamine-rhodamine (Jackson ImmunoResearch). Dilute 1/200.
Glass coverslips
Mounting media: gelmount, or "Vectashield" (from Vector Laboratories, Inc.)
Confocal microscope and associated software

Staining of Free-Floating Thick Sections. The materials are the same as for staining of sections on glass slides except:

The humidity chamber is not needed.
Reagents containing Triton X-100 are prepared with 0.3% Triton X-100 instead of 0.1%.
24-well tissue culture dishes are needed.
A small paint brush is needed.
Glass slides are needed to put sections onto after staining.

Methods

Recombinant FIV Particle Production

We routinely produce recombinant FIV particles utilizing the triple plasmid system and constructs described by Johnston *et al.*[19] (Fig. 1). This system of particle production involves concurrent transfection of TSA201 cells with three plasmids, followed by harvest of particle-containing culture media, and concentration of particles. Below are the steps for preparing 3 ml of concentrated vector particles from an 18 plate (150 mm diameter) transfection.

1. Seed TSA201 cells into 18 150-mm diameter flat-bottom tissue culture dishes at a density of 10^7 cells per dish.
2. The next day, add 34 ml of HBS to two 50-ml conical tubes. The HBS should be at room temperature.

3. Add 225 μg of the packaging plasmid, 337.5 μg of the vector plasmid, and 112.5 μg of the envelope plasmid to each HBS-containing tube and vortex well.

4. Slowly add 1.7 ml of 2.5 *M* $CaCl_2$ to each tube while slowly vortexing or shaking the HBS-plasmid mixture. $CaCl_2$ should be at room temperature.

5. Let the solution stand for 25 min to allow precipitate formation. The solution should appear slightly translucent or cloudy.

6. Add both tubes of precipitate directly to 200 ml of DMEM. Briefly mix.

7. Aspirate off the medium from the cells (9 plates at a time).

8. Gently pipette the transfection solution onto the cells (15 ml per dish), and return the cells to the incubator.

9. Four to 6 h after transfection aspirate off the medium and provide 15 ml of fresh DMEM-10 per dish.

10. Collect the medium (containing vector particles) at 24, 36, and 72 h, replacing this medium with fresh DMEM-10 at the 24 and 36 h time points. At each collection, filter the medium through a 0.45 μm bottle-top filter, and store short-term at 4°, or long-term in 50 ml aliquots at −80°.

11. Just prior to intended use, concentrate the particles by centrifuging the collected medium at 4° for 16 h at 7400*g* (7000 rpm in the Sorvall centrifuge with the SLA 1500 rotor). Carefully pour off the supernatants, drain well, and resuspend particles in lactose buffer. We typically resuspend the particles produced from an 18-plate transfection into a total 3 ml volume.

Comments on FIV Vector Particle Production

1. We have found that transfections work best with CsCl-purified plasmids.

2. We routinely suspend our concentrated particles in PBS/lactose as this buffer is physiological and acceptable for *in vivo* use, and the lactose has a stabilizing effect. However, other buffers maintaining similar pH and salt concentrations may be suitable alternatives. These include saline, TNE (50 m*M* Tris-HCL, pH 7.8, 130 m*M* NaCl, 1 m*M* EDTA), and culture medium.

3. Lentivirus vector-containing culture media suffers minimal loss in transduction titer when stored at −80°, while centrifuge-concentrated vector loses approximately one log in titer after freezing. Substantial loss of titer of concentrated preparations is also observed within 24 h of storage at 4°. Thus we routinely concentrate the virus immediately prior to use.

Determination of Particle Concentration and Transduction Titers

Standardized methods of determining the concentration of FIV-based lentivirus particles and the concentration of transduction-competent particles within lentiviral preparations have not yet been established. An ELISA assay can be used to

measure FIV p24 nucleocapsid antigen,[19,30] as an indicator of particle concentration. For determination of transduction titers, we transduce HT-1080 cells with serially diluted particles, followed by quantification of transduced cells either by staining and counting the transgene-expressing cells, or by quantitative PCR detection of pro-vector DNA sequences.

Determination of Transduction Titer by X-Gal Staining

1. One day prior to transduction, seed a 6-well flat-bottom plate with 2 million HT-1080 cells per well in DMEM-10.
2. For transduction, make a 10-fold dilution series of concentrated FIV as follows. Place 1.485 ml of DMEM-2/polybrene in first tube and 1.35 ml in tubes 2 through 6. Add 15 μl of virus to the first tube and vortex. Transfer 150 μl from the first to the second tube and vortex, and so on for the remaining tubes.
3. Remove medium from wells. Add 1 ml of each dilution to separate wells. For wells #1 through 6, the dilution factors will thus be 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7. Return the cells to the incubator.
4. Incubate the HT-1080 cells for 72 h, then feed with 1 ml of DMEM-10.
5. Incubate a further 24 h. Rinse monolayers with PBS, and then fix with 1% glutaraldehyde for 5 min at room temperature.
6. Wash once with PBS and add enough X-Gal solution to cover the cells.
7. Incubate 6 h in the dark at 37° without CO_2.
8. Rinse the wells 2× with PBS, and add PBS to cover the cells.
9. Using an inverted microscope, count the number of β-galactosidase-expressing (blue) cells in each well. The first two or three wells will often have too many positive cells to count. Doublets or small clusters of cells are counted as one, as they likely originated by division of a single transduced cell.
10. For each well, multiply the total blue cell count by the dilution volume (1 ml) and by the dilution factor. Determine the mean of all the wells. This number represents the transducing units per ml (TU/ml) of concentrated virus. Using this method, our concentrated FIVβgal preparations typically contain 5×10^7 to 10^9 TU/ml.

Determination of Transduction Titer by Quantitative PCR. A PCR-based assay system for titering the FIV vector is described elsewhere.[21] Briefly, HT-1080 target cells are transduced with serial dilutions of FIV vector preparations. The transduced cells are collected 48 h posttransfection, and genomic DNA is extracted according to standard techniques. Total genomic DNA is quantified by staining with Hoechst dye H33258 and comparison with calf thymus DNA standards, using the CytoFluor II fluorometer (PerSeptive Biosystems, Framingham, MA). Quantitative PCR is performed on 100 ng of each DNA sample, employing a PE ABI

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Prism 7700 system (Perkin-Elmer Corp., Norwalk, CT) and a synthetic oligonucleotide primer set directed against FIV packaging signal sequences yielding an 80-bp product. The resulting fluorescence is used to determine the provector copy number for each HT-1080 DNA sample, and from this the transducing units per ml (TU/ml) of the original concentrated virus is then calculated. Based on the quantitative PCR method, our concentrated FIV preparations typically contain 10^8 to 5×10^9 TU/ml.

Comments on Titering. Titering of transduction-competent particles by staining and counting of transgene-expressing cells can be applied only when a suitable staining method is available. X-Gal staining as described above is a simple and reliable method for detecting β-galactosidase-expressing cells. For other transgene products, immunostaining methods can be applied, assuming there is a suitable antibody available and the HT-1080 cells have low to no endogenous expression of the antigen. X-Gal staining or immunohistochemistry is useful for titer comparison between preparations of the same FIV vector. However, it is not recommended for comparisons between vector preparations carrying different transgenes, as the sensitivities of staining procedures for different transgene products may vary. Titering by PCR requires access to a quantitative PCR system, and effort to optimize the procedure, but once this system is established it can be applied to FIV vectors encoding any transgene. Both titering methods are based on transduction of HT-1080 cells. HT-1080 cell transduction readily occurs with VSV-G-pseudotyped FIV. However, HT-1080 cell transduction may be less efficient if the FIV vector is pseudotyped with a different envelope. Many envelope proteins exhibit restricted cellular tropism, and it might be necessary to identify more suitable cell lines for determining transduction titers of alternate pseudotypes.

Pseudotyping with Alternative Envelopes

We have described here the production of a lentivirus vector pseudotyped with the VSV-G envelope protein. VSV-G lends enhanced structural stability to vector particles, allowing for concentration by ultracentrifugation with minimal loss of infectivity.[27] In addition, VSV-G reportedly mediates viral entry via interaction with a membrane phospholipid component,[31] rather than with a specific cell surface receptor protein, and this imparts VSV-G-pseudotyped vectors with an extremely broad host-cell range, including cells of nonmammalian species.[27] These features have resulted in the common use of VSV-G for pseudotyping MLV and lentiviral gene transfer vectors.

However, there can be disadvantages to using the VSV-G envelope. Despite the wide tropism, VSV-G pseudotyped vectors do not always mediate efficient transduction. For example, VSV-G pseudotyped FIV was unable to transduce polarized airway epithelial cells when applied to the apical surface.[21] Furthermore,

[31] P. Mastromarino, C. Conti, P. Goldoni, B. Hauttecoeur, and N. Orsi, *J. Gen. Virol.* **68**, 2359 (1987).

the observations that VSV-G can be toxic to cells,[27] and that VSV-G pseudotyped vectors are inactivated upon exposure to human serum,[32] may limit the clinical application of VSV-G-pseudotyped vectors. Lastly, particularly for direct *in vivo* applications, widespread tropism can be a drawback when it is desirable to restrict transduction to specific cell types or tissues.

Several reports describe successful pseudotyping of HIV-based lentiviral vectors with alternative envelope proteins. Early HIV-1-vectors were pseudotyped with the MLV amphotropic envelope,[33,34] and the human T-cell leukemia virus envelope,[35] as well as the VSV-G envelope glycoprotein.[34] HIV vectors incorporating envelope glycoproteins from Marburg or Ebola viruses have been produced and show a wide range of infectivity.[36]

Attempts at pseudotyping can also meet with failure. Efficient incorporation of envelope proteins into particles depends on appropriate interaction of cytoplasmic envelope sequences with encapsidated genomes. Maedi-visna virus envelope constructs were unable to pseudotype MLV- or HIV-derived vector particles.[37] Pseudotyping of an HIV vector with the gibbon ape leukemia virus (GaLV) envelope glycoprotein was similarly unsuccessful.[38] However, use of a chimeric construct substituting the cytoplasmic tail of the GaLV envelope with that of the MLV amphotropic envelope protein resulted in the formation of infective GaLV-pseudotyped HIV vectors.[38]

Since FIV and HIV are related lentiviruses, successful pseudotyping of HIV with the aforementioned envelope proteins may predict similar results with FIV vectors. Preliminary studies in our laboratory indicate that FIV vectors can be pseudotyped with unmodified envelope proteins from amphotropic MLV, Marburg virus, and Ross river virus (unpublished observations), and we are currently testing these FIV pseudotypes for cell tropisms in the CNS. Also worthy of mention are a few alternative envelopes described in the literature that, although not reported in combination with lentivirus vectors, have successfully been used to pseudotype MLV vectors. Lymphocytic choriomeningitis virus envelope-pseudotyped MLV particles were structurally stable, withstanding concentration by ultracentrifugation, and exhibited cross-species tropism.[39] Pseudotyping of MLV with the Sendai

[32] N. J. DePolo, J. D. Reed, P. L. Sheridan, K. Townsend, S. L. Sauter, and D. J. Jolly, *Mol. Ther.* **2,** 218 (2000).

[33] K. A. Page, N. R. Landau, and D. R. Littman, *J. Virol.* **64,** 5270 (1990).

[34] J. Reiser, G. Harmison, S. Kluepfel-Stahl, R. O. Brady, S. Karlsson, and M. Schubert, *Proc. Natl. Acad. Sci. U.S.A.* **93,** 15266 (1996).

[35] N. R. Landau, K. A. Page, and D. R. Littman, *J. Virol.* **65,** 162 (1991).

[36] S. Y. Chan, R. F. Speck, and M. C. Ma, *J. Virol.* **74,** 49233 (2000).

[37] U. Zeilfelder and V. Bosch, *J. Virol.* **75,** 548 (2001).

[38] J. Stitz, C. J. Buchholz, M. Engelstadter, W. Uckeret, U. Bloemer, I. Schmitt, and K. Cichutek, *Virology* **273,** 16 (2000).

[39] H. Miletic, M. Bruns, K. Tsiakas, B. Vogt, R. Rezai, C. Baum, K. Kuhlke, F. L. Cosset, W. Ostertag, H. Lother, and D. van Laer, *J. Virol.* **73,** 6114 (1999).

virus fusion protein imparted a restricted tropism for asialoglycoprotein receptor-bearing cells, indicating promise for hepatocyte-directed gene delivery.[40]

The successful production of FIV-based vectors pseudotyped with an alternative envelope may simply involve following the steps outlined above, with a plasmid encoding high-level expression of the alternative envelope protein used in place of the VSV-G envelope plasmid. High-titer production of particles that display appropriate cell tropism would suggest successful pseudotyping. Low titers may indicate poor incorporation of envelope proteins into the particles, or poor receptor expression on the TSA201 or HT-1080 cells. The former problem of incompatibility may be overcome with selective mutations in the envelope protein or construction of chimeric envelopes, whereas the latter problem would necessitate usage of more appropriate cells lines in place of TSA201 and/or HT-1080 cells.

Vector Injection into Adult Mouse Striatum

We have studied the potential of gene transfer to the CNS for treatment of the neurological aspects of lysosomal storage diseases using the MPS VII mouse model. MPS VII is caused by a deficiency of β-glucuronidase, a soluble lysosomal enzyme involved in the degradation of glycosaminoglycans.[4] Like other lysosomal proteins, β-glucuronidase can be secreted and taken up by neighboring cells.[41,42] In the MPS VII (β-glucuronidase-deficient) mouse a single intra-striatal injection of most vectors encoding β-glucuronidase results in local transduction, yet the enzyme penetrates much of the injected hemisphere to provide widespread correction of pathology in both glia and neurons.[43] Vectors encoding the reporter protein bacterial β-galactosidase (a nonsecreted protein) are useful for discerning the vector transduction volume and cell types transduced (see "dual immunofluorescent staining").

Here we describe the steps involved in intrastriatal injection of FIVβgal. Sterile techniques are adhered to for brain injections. The bench space is covered with clean underpads (blue chucks), the surgical instruments are autoclaved, the mouse is prepped with iodine, and the syringe/needle is flushed and incubated with 70% ethanol and rinsed with sterile PBS prior to use. The instruments are dry-sterilized between animals.

1. Preprogram the pump for the appropriate delivery volume and rate.

[40] M. Spiegel, M. Bitzer, A. Schenk, H. Rossmann, W. J. Neubert, U. Seidler, M. Gregor, and U. Lauer, *J. Virol.* **72**, 5296 (1998).
[41] R. M. Taylor and J. H. Wolfe, *Exp. Cell Res.* **214**, 606 (1994).
[42] P. Moullier, V. Marechal, O. Danos, and J. M. Heard, *Transplantation* **56**, 427 (1993).
[43] A. Ghodsi, C. Stein, T. Derksen, G. Yang, R. D. Anderson, and B. L. Davidson, *Hum. Gene Ther.* **9**, 2331 (1998).

2. Anesthetize mice with ketamine/xylazine mix (0.1 ml per 10 g body weight), injected ip. Full anesthesia is achieved in approximately 10 to 15 min. Anesthesia is assessed by firmly pinching a toe; if the mouse exhibits a pedal reflex, anesthesia is not adequate and a second (1/3) dose of ketamine/xylazine is given.

3. Apply eye ointment to eyes. This helps prevent drying, as mice do not blink while under anesthesia.

4. Shave the dorsal aspect of the head and wipe with iodine tincture (be very careful to avoid eyes).

5. Using a scalpel, make a midline sagittal incision through the scalp over the skull to reveal the coronal, sagittal, and lambdoid sutures.

6. Lay the mouse on a small box (or something similar), to bring it up to an appropriate height for the mouse adaptor on the stereotaxic instrument. Firmly secure the head by means of the adaptor palate bar and the nose clamp. Adjust the angle of the palate bar to maintain the skull on a level plane.

7. With the (empty) syringe set into the injector unit, identify bregma as the zero coordinate. For a striatal injection, locate coordinates 0.4 mm rostral and 2.0 mm lateral to bregma, and mark the skull using an ultrafine-tip marker.

8. Remove the syringe, and drill small burr hole through the skull at the mark, being careful to maintain the integrity of the dura.

9. Draw vector into the syringe and set the syringe into the injector unit. Return the syringe to the set coordinates. Prior to lowering the syringe, "fast inject" just until a drop of vector is seen at the needle tip, to ensure proper syringe loading.

10. Lower the syringe through the burr hole until the tip of the needle touches the dura. Slowly insert the needle into the brain parenchyma to a depth of 3.0 mm. Start the microprocessor-controlled pump (preprogrammed to deliver 5 μl at a maximal rate of 500 nl per minute).

11. After vector delivery, leave the needle in place for 5 min, then slowly withdraw (over 5 min).

12. Remove the mouse from the stereotactic apparatus and close the incision with 4.0 silk suture.

13. Inject the mouse with 1 ml of lactated Ringer's subcutaneously to provide hydration during recovery.

14. Place the mouse in a cage with absorbent bedding, warmed by a lamp (75 watt bulb) 2.5 feet above the cage. Drape half the cage with a towel to allow the mouse voluntary escape from the warmth.

15. Monitor the mouse until it is ambulatory, then return it to animal housing.

Comments on Vector Injection into the Striatum. Consultation of a mouse brain atlas is necessary for identification of the skull sutures, bregma, and appropriate injection coordinates. Visualization of bregma may be facilitated by use of a dissecting microscope. Also, use of a dissecting microscope during injection allows visualization of the underlying dura while drilling, reducing the chance of puncture.

Vector Injection into Adult Mouse Cerebellum

The spinocerebellar ataxias (SCA) and other degenerative diseases of the cerebellum are potentially treatable by gene transfer if sufficient numbers of the affected neuronal types can be transduced. Cerebellar Purkinje cells are particularly affected, and as these neurons provide neuronal output for cerebellar control of movement, delivery of a therapeutic gene to these neurons may prevent Purkinje cell dysfunction/death and restore motor function. We have found that one injection of recombinant FIV encoding the reporter β-galactosidase into a cerebellar lobule transduces close to 100% of the Purkinje cells in that lobule.[14,44] Thus an FIV vector encoding a therapeutic molecule has potential clinical value.

For injection of a vector into a cerebellar lobule of an adult mouse, the procedure is essentially as outlined above for "injection into the striatum," with the following modifications. The scalp incision is more posterior with the lambdoid suture centrally revealed. The syringe is cemented with a pulled glass microcapillary tube, rather than a stainless steel needle. This type of needle is very fine, with exterior and interior diameters smaller than those of a 33-gauge stainless steel needle. The cerebellum is organized as a repeated lobular structure. However, the orientation of the lobules may differ between animals. Therefore, rather than using anterior–posterior and lateral coordinates, we choose a lobule in which to inject, then lower the needle into the brain parenchyma at a depth of 1 or 2 mm to dispense the vector (1 to 2 μl) into either the cerebellar cortex or the deep cerebellar nuclei, respectively.

Animal Perfusion/Fixation

The perfusion apparatus should be prepared prior to anesthetizing the first mouse. Set up the pump such that the rubber tubing draws PBS from a container and flushes it out the other end, which is attached to a 23 or 25 gauge butterfly i.v. catheter. Flush the line with PBS to remove all air bubbles from the circuit.

1. Anesthetize the mouse with 0.15 ml of ketamine/xylazine mix per 10 g of body weight, administered i.p. Once deep anesthesia is achieved (see above), place the mouse on its back on a Styrofoam board and tape each paw down.
2. Wet the fur over the abdomen and thorax with 70% ethanol. Using scissors, make a midline cut through the abdominal wall up from the intestinal area to just below the diaphragm, being careful to avoid cutting the underlying viscera. Continue the cut laterally, through the diaphragm (which causes an immediate tension pneumothorax), then up through the ribcage to expose the heart.
3. Using fine scissors, make a small snip in the right atrium.

[44] J. M. Alisky, S. M. Hughes, and S. L. Sauter, *NeuroReport* **11**, back cover (2001).

4. Quickly insert the i.v. catheter into the left ventricle, which can be done easily by poking through the apex of the heart.

5. Turn on the perfusion pump and flush PBS through the mouse, running the blood volume out through the cut right atrium. For a mouse, about 20–30 ml of PBS are sufficient to flush the blood volume out, which is evident by the rapid clearing of the liver from bright red to tan or brown.

6. At this point, turn off the pump and move the hose from the PBS into the paraformaldehyde. It is very important to turn off the pump while changing solutions or air emboli may be introduced that block penetration of the fixative.

7. Restart the pump and run 30–50 ml of paraformaldehyde through the mouse. Adequate fixation is indicated by the occurrence of rapid rigor mortis, due to protein cross-linking. If the mouse is limp, try carefully repositioning the tip of the needle within the left ventricle. Common errors include positioning of the needle bevel in the heart wall or entering the left atrium. Usually repositioning will save the perfusion.

Comments on Animal Perfusion/Fixation. For perfusion/fixation we use a peristaltic pump, which mimics the normal cardiac physiology, to provide thorough permeation of fixative without incurring tissue damage. Syringes or gravity (hanging solutions from the ceiling or from a high shelf) can also be used to deliver the fixative.

Removing the Brain

1. Following perfusion/fixation, decapitate the mouse with a razor blade or scalpel.

2. Use a blade to score the skull anterior to the olfactory bulbs and along the sagittal suture.

3. Using Ronguers, grasp and pull off pieces of skull from around the foramen magnum and surrounding the cerebellum. Continue pulling off pieces of skull until the brain is fully exposed laterally and as far anterior as the olfactory bulbs. Note that this procedure must be done carefully, to avoid damage to the underlying brain. A dissecting microscope may allow better visualization.

4. Use forceps and scalpel to carefully remove remaining dura from the brain surface. Scoop out the brain with a small curette (spoonlike instrument) or similar tool. The adhering olfactory nerves, optic nerves, and cranial nerves should give way with gentle pressure; if not, small scissors can be used.

5. Immerse the brain in paraformaldehyde and postfix overnight at 4°.

6. Transfer into 30% sucrose/PBS. The brain floats initially, but sinks as sucrose diffuses into the tissue. Place at 4° and allow the brain to sink. An intact mouse brain requires approximately 36 h to sink. Permeation of tissue with sucrose serves as a cryoprotectant.

7. Place the brain in a plastic mold. Cover the brain with OCT, and set the mold in a shallow dry ice/95% ethanol bath (do not allow ethanol to mix with the OCT), until the OCT is frozen (whitens upon freezing). Note that sections are cut from the bottom face of the block. Therefore before freezing, the brain can be cut first with a razor blade in the plane desired for sectioning (coronal or sagittal) and the pieces placed with cut sides down in the mold, providing a flat surface for cryosectioning. Alternatively, the whole brain can be immersed in OCT and held with forceps in a desired position until the OCT begins to harden around it.

8. Store the OCT-blocked brain at −80° until it can be cryosectioned.

Tissue Sectioning

1. Peel the plastic mold away from the OCT-blocked brain.

2. Using a cryostat, cut sections (from 8 to 50 μm thick) off the block.

3. If sections are 20 μm or less in thickness, they can be captured directly from the cryostat stage onto glass slides. Two to three sections can be placed on one slide. Slides are kept in the cryostat until the brain has been completely sectioned. The slides are transferred to slide boxes and stored at −20°.

4. For thicker sections (20 to 50 μm), use forceps to grab an edge of the section, and quickly place into a well of a 24-well tissue culture dish with PBS. Store these sections in PBS (short-term) or in PBS/azide (long-term), at 4°.

Dual Immunofluorescent Staining

We routinely use dual immunofluorescent staining and confocal microscopy for examination of transduced cells. Primary antibodies with specificity for neurons or glia can be used along with an antibody specific for the transgene product. We present methods for dual staining of β-galactosidase and NeuN (a marker common to most neuronal types), β-galactosidase and GFAP (a type II astrocyte marker), and β-galactosidase and calbindin (a common marker for cerebellar Purkinje neurons).

The following staining procedure is described for staining of 8 to 20 μm thick cryosections on glass slides. The procedure can be adapted to thicker sections (20 to 50 μm) (see "modifications for staining of free-floating sections").

All incubations are performed in a humidity chamber (which can be as simple as wet paper towels in a closed plastic container), and unless otherwise stated, incubations are at room temperature. Also, to minimize photobleaching of fluorochromes, all incubations subsequent to the addition of fluorochrome-labeled antibodies should be done in the dark.

1. Bring cryosections on glass slides to room temperature, about 10 min. This dries the sections and sticks them firmly to the slides.

2. Encircle sections with a PAP pen. Reagents are subsequently applied to slides such that the tissue is completely covered, but the reagent is held within the PAP-defined circle. Usually 100 μl volumes are sufficient to cover one section.

3. Pipette PBS onto slides and incubate for 5 min, to clear the tissue of OCT.

4. Aspirate off the PBS and add Block onto slides. Incubate for 1 h at room temperature.

5. Aspirate off Block and add primary antibodies (diluted in primary diluent). Incubate overnight at 4°.

6. Aspirate off primary antibodies and wash slides 3× with wash buffer, 10 min each.

7. Aspirate off wash and add fluorochrome-labeled secondary antibody(s). Incubate for 1–2 h.

8. Aspirate off secondary antibodies and wash slides 3× with wash buffer, 10 min each.

9. Aspirate off wash buffer and wash 1× with PBS, 10 min.

10. Aspirate off PBS. Add a small volume of mounting medium, and place a glass coverslip over the tissue. Excess mounting medium is removed by inverting the slide onto absorbent paper and applying gentle pressure. Analyze by confocal or standard fluorescence microscopy. Store in dark.

Modifications for Staining of Free-Floating Sections. The following modifications are applied for staining of thick (20 to 50 μm) free-floating sections.

1. Sections are stained (steps 3–9 above) free-floating in wells of a 24-well tissue culture dish. Sections are transferred to fresh wells for each step (use paint brush to transfer).

2. The Triton X-100 in the reagents is increased to 0.3%.

3. The primary antibody incubation time is extended to 48 h.

4. After staining, the sections are placed onto glass slides using a paintbrush. The sections are then coverslipped as described above.

Comments on Immunofluorescent Staining. The above staining procedure was optimized for FIV vector-transduced murine brain. It is important to emphasize that preliminary staining should be performed independently on each antibody to determine optimal concentrations (which can vary from lot to lot or with storage), and that appropriate control stainings be performed to confirm specificity. Negative controls should include concurrent staining of sections with (1) isotype control antibody in place of the antigen-specific primary antibody; and (2) secondary antibody alone (no previous addition of primary antibody). Fluorescence on these sections results from nonspecific binding and indicates that similar nonspecific binding is also occurring on the noncontrol (test) sections, making it difficult to distinguish true antigen-specific staining. Nonspecific binding can be reduced

or eliminated by titrating down the antibody concentrations and/or modifying the block. When the primary antibody is of the same species as the tissue, the antibody may bind "nonspecifically" to Fc receptor-bearing cells. We have not observed this to be a problem with naive or FIV-injected murine brain, but we have observed high background staining in association with inflammation incurred by other vectors. In this case, it is wise to use mouse-derived primary antibodies directly conjugated to fluorochromes, and to include normal mouse serum in the block.

Analysis of Staining by Fluorescence Microscopy

To determine which cell types are transduced, the stained slides are analyzed first by standard upright fluorescence microscopy to evaluate the quality of the stains, and then by confocal microscopy. With confocal microcopy, fluorescence emission is collected from a subcellular plane (typically 0.3 to 0.5 μm) within a

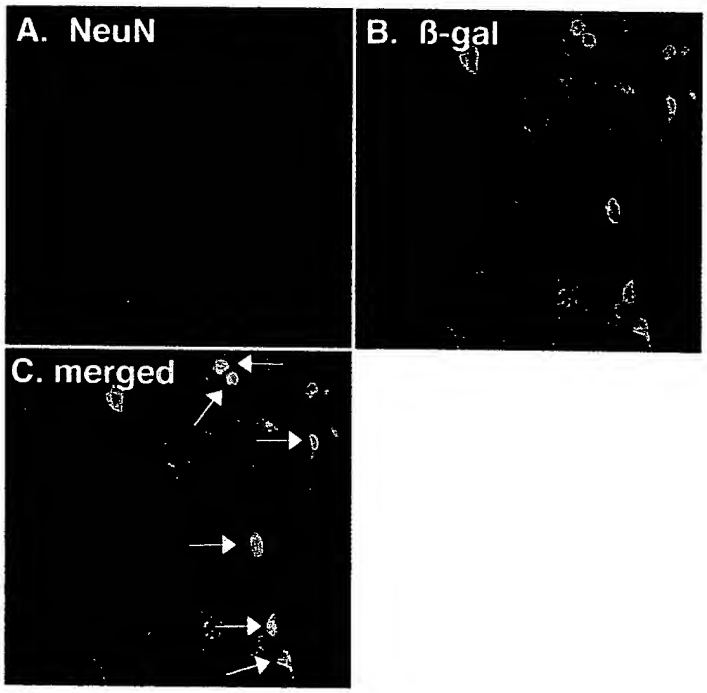

FIG. 2. Dual immunofluorescent staining for β-galactosidase and neurons. FIVβgal was injected into the striatum of a mouse brain. Eight weeks later, 50-μm coronal brain sections were dual stained with a neuronal-specific antibody (A, NeuN, red fluorescence) and a β-galactosidase-specific antibody (B, β-gal, green fluorescence). Overlapping red and green fluorescence appears yellow in the merged images (C) and identifies β-galactosidase-expressing transduced neurons (white arrows).

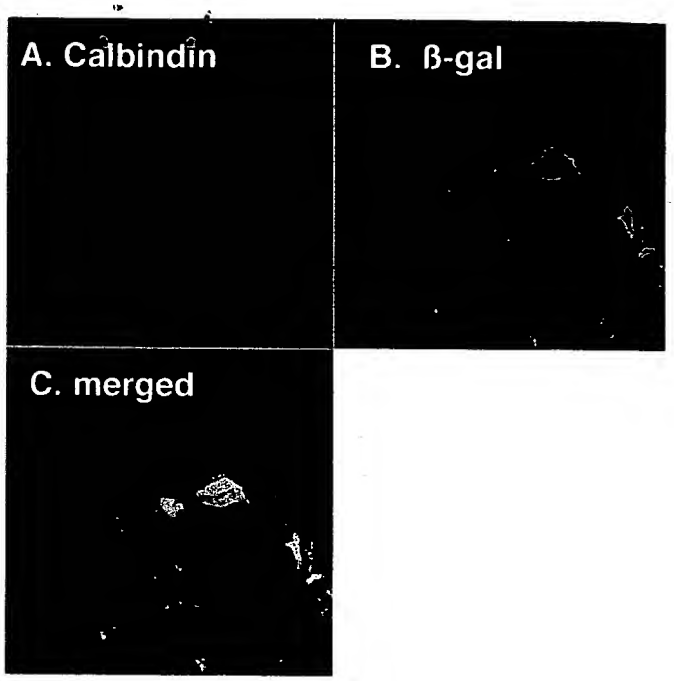

FIG. 3. Dual immunofluorescent staining for β-galactosidase and Purkinje cells. FIVβgal was injected into a cerebellar lobule of a mouse brain. Six weeks later, 50-μm coronal brain sections were dual stained with a calbindin-specific antibody (A, calbindin, red fluorescence), to detect the calbindin positive Purkinje neurons, and a β-galactosidase-specific antibody (B, β-gal, green fluorescence) to detect FIVβgal-transduced cells. Overlapping red and green fluorescence appears yellow in the merged images (C) and identifies β-galactosidase-expressing transduced Purkinje cells.

tissue section. This allows the user to unambiguously determine whether the signals from two different fluorochromes are arising from the same cell. Collection of a series of emission data from top to bottom of the tissue section allows for three-dimensional reconstruction and morphological assessment of the stained cells. Procedures for use of a confocal microscope and analysis of the data vary with the microscope and associated software.

When tissues are dual stained with one antibody that is FITC- or Alexa 488-conjugated (green fluorescence) and another that is rhodamine or CY-3-conjugated (red fluorescence), colocalization of signals appears yellow. Figure 2 shows colocalization of β-galactosidase and the neuronal cell marker NeuN in striatal neurons. Figure 3 shows colocalization of β-galactosidase and calbindin in cerebellar Purkinje cells.

Concluding Remarks

Gene transfer to the CNS for the treatment of neurodegenerative diseases is gradually progressing toward clinical application, as development of vectors advances. Successful therapy will ultimately depend on the use of a suitable vector and an effective therapeutic transgene. FIV-based vectors mediate stable gene transfer to cerebral neurons and cerebellar Purkinje cells and provide a sound basis for the further design of vectors applicable to human CNS disorders.

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